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Immunologic evaluation of 10 different adjuvants for use in vaccines for chickens against highly pathogenic avian influenza virus

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ABSTRACT

Avian influenza viruses (AIV) are a threat to poultry production worldwide. Vaccination is utilized as a component of control programs for both high pathogenicity (HP) and low pathogenicity (LP) AIV. Over 95% of all AIV vaccine used in poultry are inactivated, adjuvanted products. To identify the best formulations for chickens, vaccines were prepared with beta-propiolactone (BPL) inactivated A/British Columbia/314514-1/2004 H7N3 LP AIV using ten commercially available or experimental adjuvants. Each vaccine formulation was evaluated for immunogenicity in chickens. Challenge studies with an antigenically homologous strain of HPAIV were conducted to compare protection against mortality and measure reductions in virus levels in oral swabs. The four best adjuvants from the studies with BPL inactivated antigen were selected and tested identically, but with vaccines prepared from formalin inactivated virus. Mineral and vegetable oil based adjuvants generally induced the highest antibody titers with 100% seroconversion by 3 weeks post vaccination. Chitosan induced positive antibody titers in 100% of the chickens, but the titers were significantly lower than those of most of the oil based adjuvants. Antibody levels from calcium phosphate and alginate adjuvanted groups were similar to those of non-adjuvanted virus. All groups that received adjuvanted vaccines induced similar levels of protection against mortality (0–20%) except the groups vaccinated with calcium phosphate adjuvanted vaccines, where mortality was similar (70%) to groups that received non-adjuvanted inactivated virus or no vaccine (60–100% mortality). Virus shedding in oral swabs was variable among the treatment groups. Formalin inactivated vaccine induced similar antibody titers and protection against challenge compared to BPL inactivated vaccine groups. These studies support the use of oil adjuvanted vaccines for use in the poultry industry for control for AIV.

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1. Introduction

Avian influenza (AI) is a highly consequential disease of poultry resulting in significant economic losses worldwide due to mortal-

ity, morbidity, culling of birds, and lost trade markets. Vaccination is used to help control AI virus (AIV) and limit losses in areas where the virus is endemic. Although vectored vaccines are available and licensed in some countries, 95.5% of the AIV vaccine used for poultry, by dose, is oil emulsion, inactivated whole virus vaccine [1]. Despite the disadvantage that this type of vaccine must be applied to each bird individually, inactivated vaccines are safe, effective, and relatively inexpensive, therefore will remain highly utilized for AIV in poultry particularly in areas where labor costs are low. Individual inoculation does have the advantage that it can ensure high coverage within the vaccinated population.

Optimal formulations of inactivated vaccines need an appropriate antigen to match the field challenge virus. However, even highly immunogenic AIV strains require adjuvants to elicit a sufficient immune response. Vaccine adjuvants are chemical substances, microbial components or proteins, that enhance the

Abbreviations: 70VG, Montanide ISA 70VG adjuvant; 71VG, Montanide ISA 71VG adjuvant; 760VG, Montanide ISA 760VG adjuvant; 780VG, Montanide ISA 780VG adjuvant; AI, avian influenza; AIV, avian influenza virus; BHI, brain heart infusion buffer; BPL, beta-propiolactone; CAP, calcium phosphate adjuvant; DPC, days post-challenge; ECE, embryonating chicken eggs; EID₅₀, 50% egg infectious doses; ELISA, enzyme linked immunosorbent assay; GEL01, Montanide GEL01 adjuvant; GMT, geometric mean titer; HA, hemagglutinin protein; HAU, hemagglutinating units; HI, hemagglutination inhibition; HP, highly pathogenic; IFA, Freund's incomplete adjuvant; IM, intra-muscular; IV, intra-venous; LP, low pathogenic; OP, oropharyngeal; NAIV, non-adjuvanted inactivated virus; NP, nucleoprotein; SPF, specific pathogen free; SQ, sub-cutaneous; WPV, weeks post vaccination.

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immune response to inactivated vaccines. An ideal adjuvant should be stable and environmentally safe, should not cause an inflammatory reaction at the injection site and must be cost effective. Numerous commercial and experimental adjuvants that fulfill most of these criteria are available, but with a few exceptions [2,3] data for adjuvants with AIV vaccines in poultry are generally lacking.

The inactivation process can contribute to producing a vaccine that induces antibody to protective epitopes by affecting protein structure. Chemical treatment is the most common method of AIV inactivation for vaccine production. Formalin and beta-propiolactone (BPL) are the most commonly utilized chemicals, but both can decrease the hemagglutination (HA) titer and reduce antigenicity of influenza virus *in vitro* because of cross-linking [4,5]. Also, BPL has been shown to affect influenza HA2 protein in a manner that inhibits fusion [6]. Since the protective epitopes for influenza A neutralization reside on the HA protein this suggests that the antigenic structure could be affected. Formalin may maintain the epitopes better [5], but residues in vaccines may reduce egg production [7]. To our knowledge there is no data demonstrating the relative effects of each of these chemicals on immunogenicity with birds *in vivo*.

The goal of this study was to identify optimal adjuvants for AIV vaccines for chickens and to compare the two most common chemical inactivation methods. To accomplish this we compared the antibody responses of birds vaccinated with the same dose of different formulations of vaccines and evaluated protection (mortality and oral virus shed) against challenge with a homologous strain of highly pathogenic (HP) AIV.

2. Materials and methods

2.1. Virus

The A/chicken/British Columbia/314514-1/2004 H7N3 low pathogenic (LP) AIV isolate was used to produce the vaccines, and a related highly pathogenic (HP) AIV isolate (antigenically homologous isolate) was used for challenge: A/chicken/British Columbia/314514-2/2004 H7N3 [8]. These isolates were selected so the vaccine could be prepared with an LP strain for safety and the challenge could be conducted with an antigenically identical but highly virulent (i.e. HP) isolate. Additionally, previous studies with these isolates have shown that they are moderately immunogenic, therefore should better discriminate between adjuvants than isolates at the low or high extremes of immunogenicity [9]. Using standard methods in specific pathogen free (SPF) embryonating chicken eggs (ECE) [10] each isolate was propagated and titrated for use as vaccine antigen, antigen for hemagglutination inhibition (HI) assay, and challenge virus.

2.2. BPL inactivation

The LPAIV (infectious allantoic fluid from embryonating chicken eggs) was inactivated by treatment with 0.1% BPL with incubation at ambient temperatures (approximately 20–23 °C) for 4–6 h with constant mixing, then was incubated overnight at 4 °C [11]. Prior to testing the antigenic content by hemagglutination assay (HA) the pH was adjusted to approximately 7.0 with sodium bicarbonate solution. The HA assay was conducted using standard procedures [12].

2.3. Formalin inactivation

The LPAIV was inactivated by treatment with 0.02% formalin with incubation at 37 °C for 18–24 h [4]. The antigenic content was quantified by standard HA assay [12].

2.4. Vaccine preparation

Each vaccine that was prepared with a commercial adjuvant was made in accordance with the manufacturers recommendations. Commercial oil based (water-in-oil) adjuvants included: Montanide ISA 70VG (70VG) (mineral oil based) (SEPPIC, Inc., Fairfield, NJ), Montanide ISA 71VG (71VG) (SEPPIC), Montanide ISA 760VG (760VG) (synthetic polymer and ester based) (SEPPIC), Montanide 780 VG (780VG) (vegetable oil based) (SEPPIC) and Montanide GEL01 (GEL01) (synthetic polymer based) (SEPPIC) (Table 1). A mineral oil adjuvant that was developed in-house was prepared as described by Stone et al. [13] (Stone adjuvant). Incomplete Freund's adjuvant (IFA) was prepared with a commercial product (Sigma-Aldrich Co, St. Louis, MO). Calcium phosphate (CAP), alginate, and chitosan adjuvanted vaccines were prepared as reported previously [14,15]. Because potency has been shown to vary between 160 and 512 HAU among different AIV isolates [9,16], we used the maximum uniform dose we could achieve taking into account the dilution effect with each adjuvant. Therefore all vaccine formulations were standardized to contain 384 hemagglutinating units (HAU) per dose.

2.5. Vaccination-challenge studies

Four-weekold SPF white leghorn chickens were obtained from Southeast Poultry Research Laboratory, USDA-ARS (SEPR) in-house flocks and were individually tagged for identification. The studies were conducted in accordance with the appropriate recommendations of the Federation of Animal Science Societies Guide for the Care and Use of Agricultural Animals in Research and Teaching 3rd Edition. This study and associated procedures were reviewed and approved by the SEPR Institutional Animal Care and Use committee. Chickens were divided into groups of 10 for the BPL inactivated vaccines and groups of 20 for the formalin inactivated vaccines (Table 1). Each vaccine was administered in 0.5 ml by the sub-cutaneous route. Control groups included: chickens that received 384 HAU of non-adjuvanted inactivated virus (NAIV) by the sub-cutaneous (SQ), intra-muscular (IM) and intravenous (IV) routes and non-vaccinated chickens.

Serum was collected from all chickens three weeks post vaccination to evaluate antibody levels then the birds were challenged with 10^6 50% egg infectious doses (EID_{50})/bird of A/chicken/British Columbia/314514-2/2004 H7N3 HPAIV in 0.1 ml by the intra-chanoal route (to simulate natural exposure). In the BPL vaccine groups all ten chickens were challenged, however in the formalin vaccine groups ten chickens (half) from each vaccine group were randomly selected for challenge. The remaining ten chickens were kept for an additional 3 weeks to evaluate antibody levels 6 weeks post vaccination.

Chickens were monitored daily for clinical signs and mortality until 14 days post challenge (DPC). Oropharyngeal (OP) swabs were collected from each chicken at 2, 4 and 7 DPC, placed in 2 ml brain heart infusion (BHI) broth with antibiotics (2 µg/ml amphotericin B; 1000 Units/ml penicillin G; and 100 µg/ml gentamicin), and stored at –70 °C until they were processed for virus testing by real-time RT-PCR (rRT-PCR) as described below.

Table 1

Vaccine formulations, routes of administration and treatment group sizes.

Adjuvant	Abbreviation	Adjuvant type	Emulsion type	Route of inoculation ^b	Number of chickens	
					BPL ^c inactivated	Formalin inactivated
Montanide ISA 70 VG	70 VG	Mineral oil	Water-in-oil	SQ	10	NA
Montanide ISA 71 VG	71 VG	Mineral oil	Water-in-oil	SQ	10	20
Montanide ISA 760 VG	760 VG	Synthetic lipophilic polymer/ester	Water-in-polymer	SQ	10	20
Montanide ISA 780 VG	780 VG	Vegetable oil	Water-in-oil	SQ	10	20
Montanide GEL 01	GEL01	Synthetic polyacrylic polymer	None, aqueous	SQ	10	NA
Stone adjuvant	Stone	Mineral oil	Water-in-oil	SQ	10	20
Incomplete Freund's	IFA	Mineral oil	Water-in-oil	SQ	10	NA
Chitosan	NA ^a	Carbohydrate (deacylated chitin)	None	SQ	10	NA
Ca Phosphate	CAP	Mineral nanoparticle	None	SQ	10	NA
Alginate	NA	Seaweed derived	None	SQ	10	NA
Non-adjuvanted virus	NA	NA ^a	NA	IM	10	NA
Non-adjuvanted virus	NA	NA	NA	IV	10	NA
Non-adjuvanted virus	NA	NA	NA	SQ	10	20
Non-vaccinated	NA	NA	NA	NA	10	20

^a NA = not applicable.^b IM = intramuscular; IV = intravenous; SQ = subcutaneous.^c BPL = beta propiolactone.

2.6. Characterization of the serological response to vaccination

Three weeks post vaccination (WPV) all chickens were bled to evaluate the antibody response to each vaccine formulation. Sera were tested by homologous HI assay (quantitative) and a commercial blocking ELISA kit, MultiS-screen (IDEXX Laboratories, Inc., Westbrook, ME) (non-quantitative). Ten chickens from each group vaccinated with the formalin inactivated antigen were not challenged and were also bled to evaluate antibody levels six weeks post vaccination. The HI assay was conducted in accordance with standard procedures [17] using the same preparations of antigen that was used to prepare the vaccines. A titer of 1:16 (1:2⁴) or above was considered positive.

2.7. RNA extraction and quantitative real-time RT-PCR

Total RNA was extracted from swab material as described by Das et al. [18] using the MagMAX 96 AI Viral RNA isolation kit (Thermo-Scientific, Waltham, MA) with the KingFisher magnetic particle processor (Thermo-Scientific). Quantitative rRT-PCR which targets the influenza M gene [19] was performed using the 7500 FAST Real-time PCR System (Thermo-Scientific), and the AgPath-ID OneStep RT-PCR kit (Thermo-Scientific) as reported previously [20]. A standard curve for virus quantification was established with RNA extracted from dilutions of the same titrated stock of virus used to inoculate the chickens and was run in triplicate.

2.8. Statistical methods

Virus titers in OP swabs were tested for statistical significance among vaccines groups on the same DPC with the Kruskal-Wallis test, Dunn's method (SigmaPlot 12.0, Systat Software, Richmond, CA). If virus was not detected in a sample, it was given the value of 0.1 log₁₀ below the qRRT-PCR test limit of detection. Antibody titers determined by HI assay were compared between vaccine groups by the Kruskal-Wallis test and Dunn's multiple comparison. A p value of ≤0.05 was considered to be significant (the term "significant" is only used when statistical testing was conducted with the data to which it refers).

3. Results

3.1. Antibody levels

Antibody responses were quantified by homologous HI which measures antibody bound to the HA protein. Among the groups vaccinated with BPL inactivated antigen there were significant differences in antibody levels at three weeks post vaccination (WPV) (Fig. 1, Table 2). The adjuvants can be roughly grouped by relative levels of antibody induction: Montanide ISA 71VG, ISA 70VG and GEL01 induced the highest levels of antibody (GMT range of 222.9–630.3), while 760VG, 780VG, the Stone adjuvant, incomplete Freund's and Chitosan induced intermediate levels (GMT

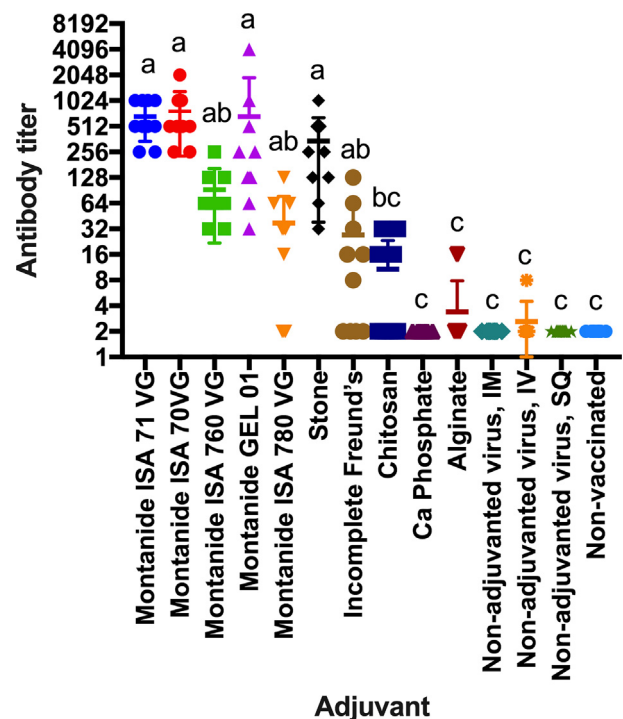


Fig. 1. Antibody titers from chickens three weeks post vaccination with beta-propiolactone inactivated antigen. Titers were determined by homologous hemagglutination inhibition assay. Error bars show standard deviation. Statistical groups are denoted with letters.

Table 2

Antibody response in chickens for different vaccine formulations determined by homologous hemagglutination inhibition (HI) assay (geometric mean titer [GMT] and percent of chickens with detectable antibody) and percent of chickens positive for detectable antibody by commercial blocking ELISA at 3 and 6 weeks post vaccination (PV).

Adjuvant	HI Titer GMT (%positive)			ELISA% positive		
	BPL ^b inactivated 3 weeks PV	Formalin inactivated 3 weeks PV	6 weeks PV	BPL inactivated 3 weeks PV	Formalin inactivated 3 weeks PV	6 weeks PV
Montanide ISA 70 VG	630.3 (100)	NA ^c	NA	100	NA	NA
Montanide ISA 71 VG	588.1 (100)	110.6 (100)	415.9 (100)	100	100	100
Montanide ISA 760 VG	74.7 (100)	21.7 (57.9)	119.4 (100)	100	87.5	90
Montanide ISA 780 VG	43.1 (70)	NA	NA	100	NA	NA
Montanide GEL 01	238.9 (100)	76.1 (100)	109.7 (100)	100	100	100
Stone adjuvant	222.9 (100)	87.4 (100)	194.0 (100)	100	95	100
Incomplete Freund's	28.5 (50)	NA	NA	100	NA	NA
Chitosan	22.6 (90)	NA	NA	100	NA	NA
Ca Phosphate	13.9 (70)	NA	NA	10	NA	NA
Alginate	10.6 (50)	NA	NA	40	NA	NA
Non-adjuvanted virus: IM ^a	8 (10)	NA	NA	30	NA	NA
Non-adjuvanted virus: IV ^a	8 (10)	NA	NA	20	NA	NA
Non-adjuvanted virus: SQ ^a	<2	<2	4	20	30	10
Non-vaccinated	<2	<2	<2	0	0	0

^a IM = intramuscular; IV = intravenous; SQ = subcutaneous.

^b BPL = beta-propiolactone.

^c NA = not applicable.

range of 22.6–74.7) (Table 2). Alginate and CAP adjuvanted vaccines induced low antibody titers (GMTs of 10.6 and 13.9 respectively) that were the statistically the same as those from NAIV inoculated groups and non-vaccinated chickens (Fig. 1, Table 2).

A subset of adjuvants was also tested with formalin inactivated antigen, with similar results to those observed with the corresponding adjuvants with the BPL inactivated antigens (Fig. 2, Table 2). At 3 WPV ISA71 VG, GEL01 and the Stone adjuvant induced the highest levels, while 760VG was lowest. All were significantly higher than antibody levels from chickens that were vac-

inated with NAIV and non-vaccinated chickens. Resources allowed for half of the chickens vaccinated with the formalin inactivated material to be held longer so they could be tested again at 6 WPV. Antibody titers in all groups except the GEL01 adjuvanted group and non-vaccinated controls had increased significantly by 6 WPV versus the titers for each adjuvant at 3 WPV (Fig. 2).

Titers from chickens vaccinated with BPL inactivated antigen were compared with titers 3 WPV from chickens vaccinated with formalin inactivated material administered with the same adjuvant. Titers from chickens vaccinated with the BPL inactivated material were significantly higher with all four adjuvants that were tested with both inactivation methods (ISA 71VG, ISA 760 VG, GEL01 and the Stone adjuvant). However, titers from the chickens vaccinated with the formalin inactivated antigen were equivalent at 6 WPV to the titers from BPL inactivated antigen at 3 WPV with all but the GEL01 adjuvant, which was still significantly lower.

A commercial ELISA measuring antibody to the viral nucleoprotein (NP) protein was also used to evaluate the number of chickens which seroconverted following vaccination. In most cases the results were identical to the HI results (Table 2), however there were a few birds which demonstrated positive antibody titers with only one of the assays. For example, a few birds receiving the NAIV material were positive for antibody by ELISA, but not the HI assay. Regardless of adjuvant, inactivation method or antibody detection method, the number of chickens which seroconverted by 3 WPV was significantly higher in adjuvanted vaccine groups than the groups which received NAIV (Table 2).

3.2. Protection against challenge

Protection from HPAIV challenge was determined by increased survival and decreased viral shedding. The percent survival by group is shown in Fig. 3. Regardless of inactivation method survival was significantly increased in all groups which received an adjuvanted vaccine, except the CAP adjuvanted group (Fig. 3). Survival was also increased in groups of chickens that received NAIV compared to non-vaccinated controls, but not significantly. Also, only two birds demonstrated clinical signs of disease, a chicken in the BPL inactivated 71VG group and one in the BPL inactivated NAIV IV group. This was not unexpected because death from HPAIV can occur rapidly in the absence of overt clinical signs and thus is not a reliable or discriminatory metric of protection in experimental HPAIV challenge.

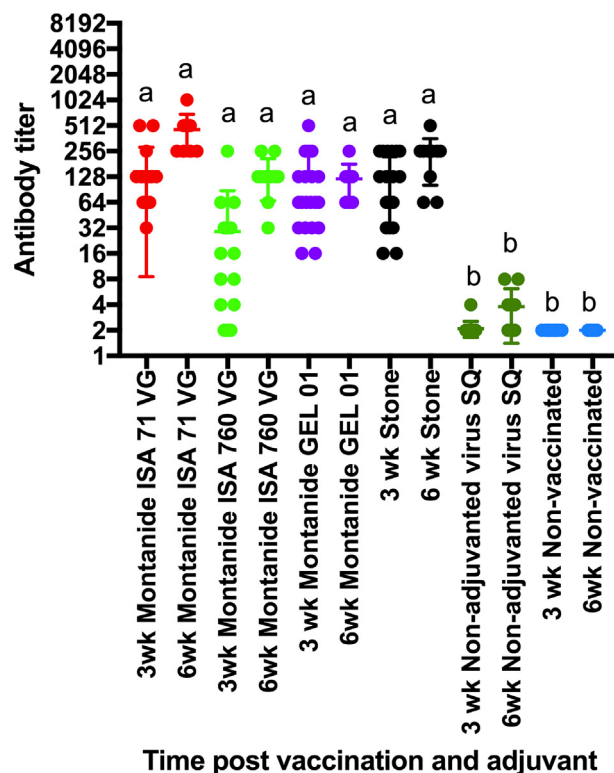


Fig. 2. Antibody titers from chickens three and six weeks post vaccination with formalin inactivated antigen. Titers were determined by homologous hemagglutination inhibition assay. Error bars show standard deviation. Statistical groups are denoted with letters.

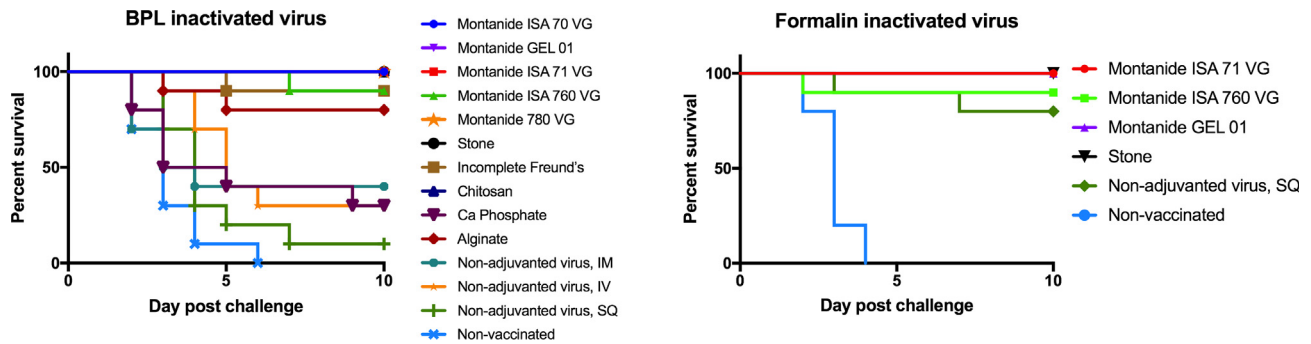


Fig. 3. Survival of chickens challenged with 10^6 50% egg infectious doses of A/chicken/British Columbia/314514-2/2004 H7N3 highly pathogenic avian influenza virus by the intra-choanal route three weeks post vaccination: (A) chickens vaccinated with vaccines prepared from antigen inactivated with beta-propiolactone; (B) chickens vaccinated with vaccines prepared from antigen inactivated with formalin.

Using BPL inactivated antigen, shedding was significantly reduced at 2DPC compared to the non-vaccinated controls in all groups which received adjuvanted vaccine, except those vaccinated with the IFA, CAP, and alginate adjuvanted preparations (Fig. 4). Viral shedding in groups which received NAIV was similar to the non-vaccinated group (the non-vaccinated group was used as the reference for shed levels). At 4DPC there were too few chickens left in the CAP, non-vaccinated, and NAIV IM administration vaccine groups for statistical analysis, however all groups that received adjuvanted vaccines, shed statistically similar levels of virus orally, which was significantly less than the NAIV administered by the SQ and IV routes. Similarly, when vaccines were prepared from formalin inactivated material all groups vaccinated with the adjuvanted vaccines shed significantly less virus orally at both 2DPC and 4DPC compared to the NAIV and non-vaccinated groups (Fig. 5). By 7DPC few vaccinated chickens were shedding and the non-vaccinated chickens were mostly dead, however a few NAIV chickens were still shedding (Figs. 4 and 5).

4. Discussion

Ten adjuvants were compared for the induction of antibody and protection against HPAIV challenge in chickens. Selected adjuvants were also tested using formalin instead of BPL to inactivate the antigen so that the effect of inactivation method on immunogenicity or protection could be evaluated. As expected, NAIV provided no immunity or protection against mortality, nor did it reduce virus shed.

With the BPL inactivated antigen, the highest antibody responses and most complete protection to disease and virus shed were observed with vaccines prepared with either 70VG or 71VG, which performed similarly to each other. Previous reports have shown that each are effective adjuvants for chickens with different viral and bacterial agents [2,3,21–25]. Although the antibody response produced by the Stone adjuvant, IFA, 760VG and GEL01 produced were lower than 70VG or 71VG, the differences were not significant and protection against mortality and reduction of shed were similar. Data for 760VG and GEL01 are lacking, but both the Stone adjuvant and IFA have been shown to work well in chickens with bacterial and viral antigens [13,26,27]. Antibody titers in the remaining 3 groups were significantly lower and with the

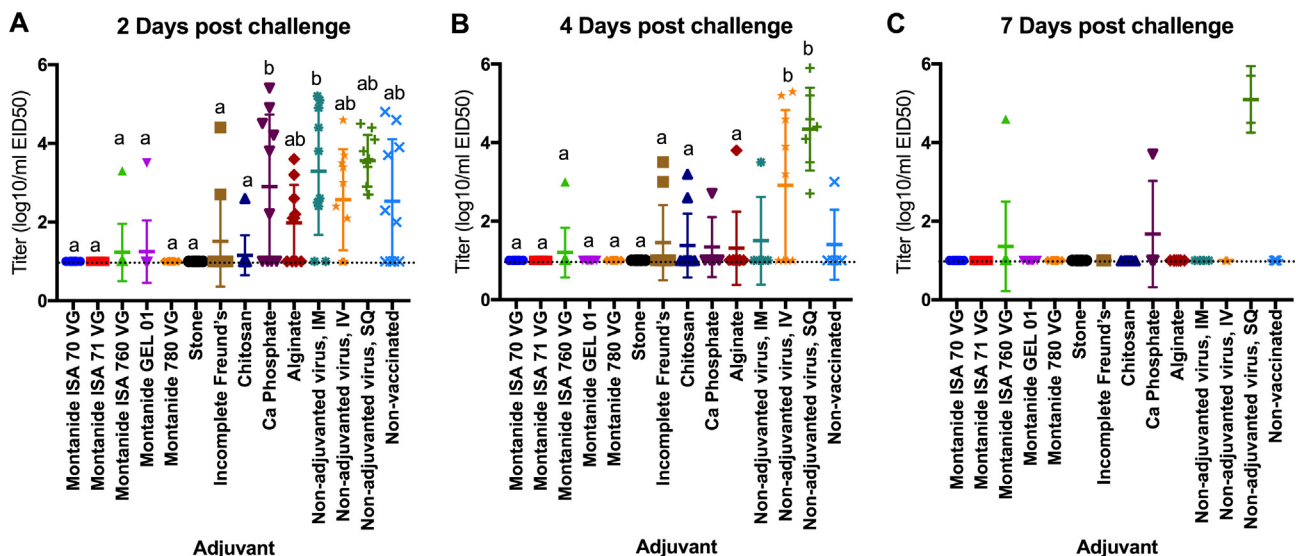


Fig. 4. Virus titers in oropharyngeal swabs from chickens to which vaccines prepared from antigen inactivated with beta-propiolactone were administered, detected by quantitative real-time RT-PCR: (A) 2 days post challenge (DPC) with 10^6 50% egg infectious doses of A/chicken/British Columbia/314514-2/2004 H7N3 highly pathogenic avian influenza virus by the intra-choanal route; (B) 4DPC; (C) 7DPC. Error bars represent mean and standard deviation; a dotted line represents the approximate limit of detection; samples where virus was not detected are shown at the limit of detection. Statistical groups are denoted with letters; groups with no letters indicate that too few birds were alive for reliable statistical analysis.

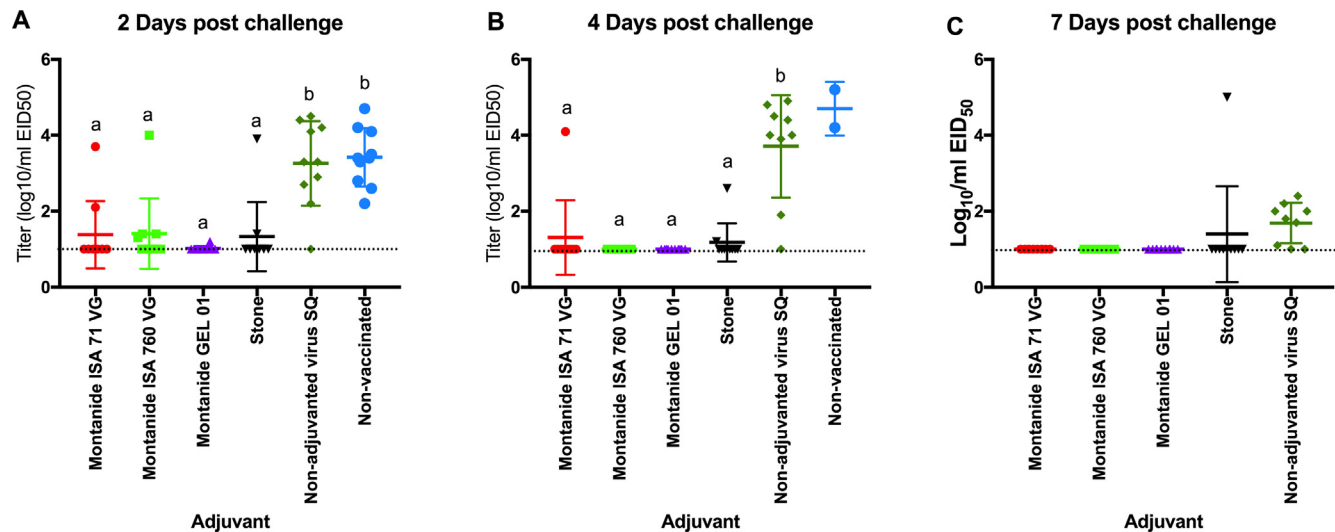


Fig. 5. Virus titers in oropharyngeal swabs from chickens to which vaccines prepared from antigen inactivated with formalin were administered, detected by quantitative real-time RT-PCR: (A) 2 days post challenge (DPC) with 10^6 50% egg infectious doses of A/chicken/British Columbia/314514-2/2004 H7N3 highly pathogenic avian influenza virus by the intra-choanal route; (B) 4DPC; (C) 7DPC. Error bars represent mean and standard deviation; a dotted line represents the approximate limit of detection; samples where virus was not detected are shown at the limit of detection. A dotted line shows the approximate limit of detection. Statistical groups are denoted with letters; groups with no letters indicate that too few birds were alive for reliable statistical analysis.

exception of 780VG, protection was also reduced to levels similar to the NAIV groups. Overall the adjuvants which performed the best were oil based and form a water-in-oil emulsion.

Chitosan is more typically used as a mucosal adjuvant with intra-nasal or oral administration [25,28–32], therefore the relatively poor immune response may have been due to a sub-optimal delivery method for this adjuvant. Although, chitosan did provide 100% protection against mortality and did reduce shed titers significantly compared to the non-vaccinated controls.

There have been few reports with alginate in avian species, but it has been tested successfully in numerous mammalian species and even fish [14,33–36]. However, it is typically used as a mucosal adjuvant or stabilizer in oral vaccines [35,37,38], although injection of experimental vaccines in mice has been successful [34]. In chickens the alginate adjuvanted vaccine did not induce high antibody levels, but was moderately protective against challenge; mortality was reduced, but the chickens shed virus levels similar to NAIV inoculated groups.

Similar to alginate, CAP has been used as an adjuvant and stabilizer in mammalian model systems [39–43], and also has been used in chickens with Newcastle disease virus vaccines [15,44]. Here CAP performed similarly to the NAIV and non-vaccinated controls, therefore would not be a viable candidate for an AIV vaccine for chickens. This contrasts a report where CAP adjuvanted vaccine induced high antibody titers in chickens with a Newcastle disease virus vaccine [44]. The difference may be due to the fact that a lower dose of antigen was used in these studies or some intrinsic property of the antigen (e.g. AIV may not bind CAP as well as Newcastle disease virus). Consistent with our results, Volkova et al., [15] did find that chitosan performed better than CAP in chickens with the Newcastle disease virus vaccine.

As a general trend all of the adjuvants except CAP and alginate significantly reduced shed at 2 and 4DPC compared to both the NAIV and non-vaccinated chickens. At 7DPC with the exception of few individual chickens, all the vaccinated chickens were no longer shedding detectable levels of virus, but the NAIV chickens that had survived were still shedding (very few non-vaccinated chickens were alive at 7DPC). As a whole, this demonstrates that all of the adjuvants, except CAP and Alginate, were able to substan-

tially reduce virus shed, which is among the most critical metrics of AIV vaccine efficacy.

There was a significant difference in antibody titers between the groups vaccinated with BPL and formalin inactivated antigens at 3 WPV, although by 6 WPV the titers were statistically the same as those of the BPL groups at 3 WPV with 3 of 4 the adjuvants. Therefore, antibody development was slower for unknown reasons. Possibly the formalin altered the antigen so that it was less immunogenic or the BPL altered the protein to be slightly more immunogenic. Also, the inactivation procedure for formalin is conducted at a higher temperature, which could have had some effect. Both chemicals have been noted to decrease HI titer with some AIV isolates and possibly alter protein structure [4–6]. However, the specific changes that may have occurred with this HA have not been characterized. Importantly, at 3 WPV, the groups that received the vaccines prepared with formalin inactivated antigen were protected against mortality and orally shed virus levels similar to the chickens that received the vaccine prepared with BPL inactivated antigen. Therefore the difference did not affect protection.

Because these are whole virus vaccines we could evaluate antibody by HI assay, which detected antibody to the HA protein and positively correlates with protection [45], and by ELISA which detects antibody to the NP. Interestingly, more birds were positive for antibody by ELISA than by HI assay from groups vaccinated with the adjuvants that did not protect, reinforcing that this ELISA does not detect antibody that correlates with protection. It's not clear if there is a mechanistic difference, where these adjuvants enhance immunity to the NP instead of the HA. There were no differences among the oil based adjuvants because seroconversion was 100% with both assays. We did not measure whether there was a difference among adjuvants in the antibody threshold for protection. Since antibody levels only has positive predictive value [45], especially with heterologous challenge, information from this homologous challenge may not directly apply to the field. It is important to recognize that the most important metric for the efficacy of a vaccine is not necessarily the antibody level it induces to the challenge virus, but the level of protection provided against mortality, disease and virus shedding.

Antibody levels can be enhanced by adjuvants, but are also related to antigen load and immunogenicity of the HA in the vaccine. We did not evaluate how robust the adjuvants were to decreasing the antigen load in the vaccine, which could decrease vaccine production costs substantially. One report has shown that the antigen in a Newcastle disease virus vaccine could be reduced as much as 100X without an impact on potency when using the ISA 70VG adjuvant [46], whether this would be true for AIV needs to be explored. We also did not specifically evaluate injection site reactions beyond visual inspection. Data have been previously reported on the safety of most of these adjuvants in the literature [15,37] or by the manufacturer. Although, a similar response is expected, further studies are needed to evaluate these adjuvants in turkeys and ducks, since vaccination is used to control influenza in these species as well. In conclusion, these studies demonstrate that the oil-based adjuvants performed better than the alternative formulations, and that BPL and formalin inactivated antigens both elicited protective immunity by 3 WPV. Whether protective immunity can be induced at an earlier time will be the subject of future studies.

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Conflict of Interest

The authors declare that they have no conflicts of interest.

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